J. Clin. Chem. Clin. Biochem.
Vol. 15, 1977, pp. 293–296

# Quantitative Determination of Glycosphingolipids in Human Plasma[1])

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(Received August 15/November 25, 1976)

**Summary:** A method is described for the quantitative isolation and estimation of the four glycosphingolipid fractions from 10 ml of human plasma. The procedure consists of acetylation of the total lipids after extraction, separation of acetylated glycosphingolipids from non-glycolipids on a Florisil column, deacetylation, dialysis in water, separation of the single fractions by thin-layer chromatography on silica gel, and quantitative analysis of each glycosphingolipid by sulfuric acid-orcinol reagent, carried out in the presence of silica gel. The concentrations of the four glycosphingolipids in human plasma derived from a group of 23 healthy subjects are presented. The procedure described is sufficiently sensitive for clinical investigation.

*Quantitative Bestimmung der Glycosphingolipide im menschlichen Blutplasma*

**Zusammenfassung:** Über eine Methode zur quantitativen Isolierung und Bestimmung der vier neutralen Glycosphingolipidfraktionen aus 10 ml menschlichem Blutplasma wird berichtet. Das Verfahren besteht aus: Acetylierung der extrahierten Gesamtlipide, Abtrennung der acetylierten Glycosphingolipide durch Säulenchromatographie an Florisil, Deacetylierung, Dialyse gegen Wasser, Auftrennung der einzelnen Fraktionen durch Dünnschichtchromatographie auf Kieselgelplatten und quantitative Bestimmung der verschiedenen Glycosphingolipide im Schwefelsäure-Orcinol-Test in Gegenwart von Kieselgel. Die ermittelten Konzentrationen der vier Glycosphingolipide in einem Kollektiv von 23 gesunden Personen werden mitgeteilt. Das beschriebene Verfahren ist empfindlich und geeignet für klinische Untersuchungen.

## Introduction

Four fractions of neutral glycosphingolipids have been isolated from human plasma and characterized: monohexosyl (glucosyl) ceramide, dihexosyl (lactosyl or galactosyl-glucosyl) ceramide, trihexosyl (galactosyl-galactosyl-glucosyl) ceramide, and tetrahexosyl (N-acetylgalactosaminyl-galactosyl-galactosyl-glucosyl) ceramide (1, 2). Abnormalities of glycosphingolipid metabolism that occur in several sphingolipidoses such as *Gaucher*'s disease, lactosyl-ceramidosis or *Fabry*'s disease and secondarily in other diseases like hyperlipoproteinemia (3, 4, 5) have led to an increasing interest in the behavior and physiological function of the glycosyl ceramides in human blood. Previous studies on the isolation of single glycosyl ceramides from human blood included a variety of chromatographic steps and required large volumes (17 l) of plasma (1). *Vance & Sweeley* (2) presented a method, including gas-liquid-chromatography, for the quantitative determination of the glycosyl ceramides in human plasma requiring 50 ml of plasma. *Saito & Hakomori* (6) introduced a quantitative isolation of total glycosphingolipids from animal cells using column chromatography on Florisil of the peracetylated glycolipids, but the estimation of the carbohydrate moiety by gas-liquid-chromatography is necessary. *Neskovic* et al. (7) described the analysis of the isolated glycolipids from brain without previous elution from silica gel by a spectrophotometric assay, employing the orcinol method of *Svennerholm* (8). This paper describes the quantitative isolation and determination of the four glycosphingolipids derived from 10 ml plasma, using among other steps, chromatography on Florisil of the acetylated glycolipids and estimation of the carbohydrate part by the orcinol reagent.

[1]) Supported by the Deutsche Forschungs-Gemeinschaft (At 8/1).

## Materials and Methods

Heparinized blood samples were obtained from 23 healthy subjects (10 males, 13 females, age: $\bar{x}$ = 32a, 22–74) after a fasting period of 12 hours. The blood samples were centrifuged for 20 min at 1700 *g* to separate the plasma.

### *Chemicals*

Orcinol, *D*(+)-galactose and *D*(+)-glucose were obtained from E. Merck AG (Darmstadt, Germany). Florisil (60–100 mesh) was purchased from Serva (Heidelberg, Germany). Reference substances of glucosyl-, lactosyl-, trihexosyl- and tetrahexosyl ceramide were generous gifts from Prof. Dr. *W. Gielen*, Biochemische Pharmakologie des Pharmakologischen Institutes, Universität Köln (Germany). Cerebroside (galactosyl ceramide) was purchased from „Applied Science Laboratories, Inc., USA", and synthetic glucosyl- and lactosyl ceramide were obtained from "Research Division Miles Laboratories, Inc., Frankfurt/Main" (Germany). All other chemicals used were analytical grade reagents obtained from commercial sources.

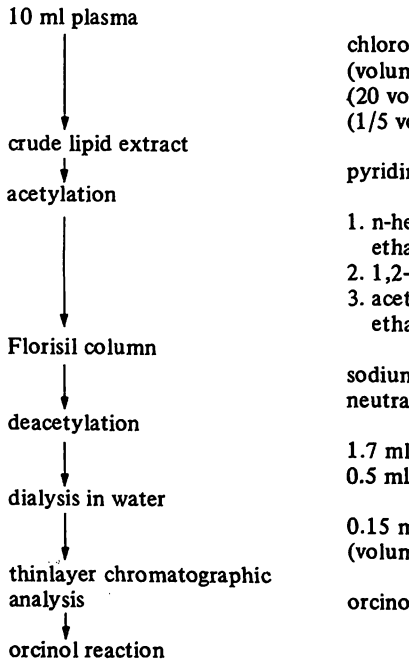
### *Orcinol reagent*

140 ml of concentrated sulfuric acid were diluted with distilled water under cooling to a final volume of 200 ml. Before use 100 mg orcinol were dissolved in 100 ml of cold diluted sulfuric acid.

## Procedure

### *Isolation of glycosyl ceramides*

Ten ml plasma were mixed with 200 ml of chloroform-methanol (volumes, 200 ml + 100 ml) (scheme 1). After standing for 2 hours at room temperature the mixture was filtered, and the residue was washed with 3 ml of chloroform-methanol (volumes, 2 ml + 1 ml). The combined extracts were mixed thoroughly with 40 ml of water, and the layers were allowed to separate overnight in the cold room. After separation the residue (crude lipid extract) was dissolved in 1.8 ml of dried pyridine and 0.6 ml of acetic anhydride. After standing at room temperature for 18 hours 300 ml of dry toluene were added. The solvents were completely evaporated. The dry residue was dissolved in 1.5 ml of n-hexane-1,2-dichloroethane (volumes, 1 ml + 4 ml) and put on a column (1 × 25 cm, capacity 9.5 g) of deactivated Florisil (magnesia-silica gel, 60–100 mesh) prepared with the same solvent. Subsequently the column was eluted stepwise with 130 ml each of the following solvents: n-hexane-1,2-dichloroethane (volumes, 1 ml + 4 ml), 1,2-dichloroethane, 1,2-dichloroethane-acetone (volumes, 1 ml + 1 ml), 1,2-dichloroethane-methanol-water (volumes, 2 ml + 8 ml + 1 ml). The acetylated glycosphingolipids were eluted with 1,2-dichloroethane-acetone. This fraction containing the acetylated glycosphingolipids was evaporated to dryness in vacuo. The residue was dissolved in 1.2 ml chloroform-methanol (volumes, 2 ml + 1 ml) and 0.3 ml of 5 g/l sodium methoxide in methanol. After standing for 30 min at 25 °C the solution was neutralized by addition of 0.6 ml ethyl acetate, evaporated to dryness, emulsified in 1.5 ml water and 0,5 ml "theoretical upper phase" (chloroform-methanol-1 g/l NaCl, (volumes, 1 ml + 10 ml)), transferred in a dialysis membrane (Visking, $\phi$ 7 mm, diameter of pores 1.5–2.0 nm, obtained from Serva, Heidelberg, Germany), and dialysed against 200 volumes of ice water overnight. The dialysed solution was evaporated to dryness in a rotatory evaporator and kept in a desiccator over phosphorus pentoxide. The dried residue was dissolved in 0.15–0.2 ml of chloroform-methanol (volumes, 2 ml + 1 ml) for analysis by thin layer chromatography. Commercially available thin-layer plates (20 × 20 cm, layer thickness 0.25 mm, silica gel 60, obtained from E. Merck, Darmstadt)




Scheme 1. Summary of glycosphingolipid isolation from human plasma.

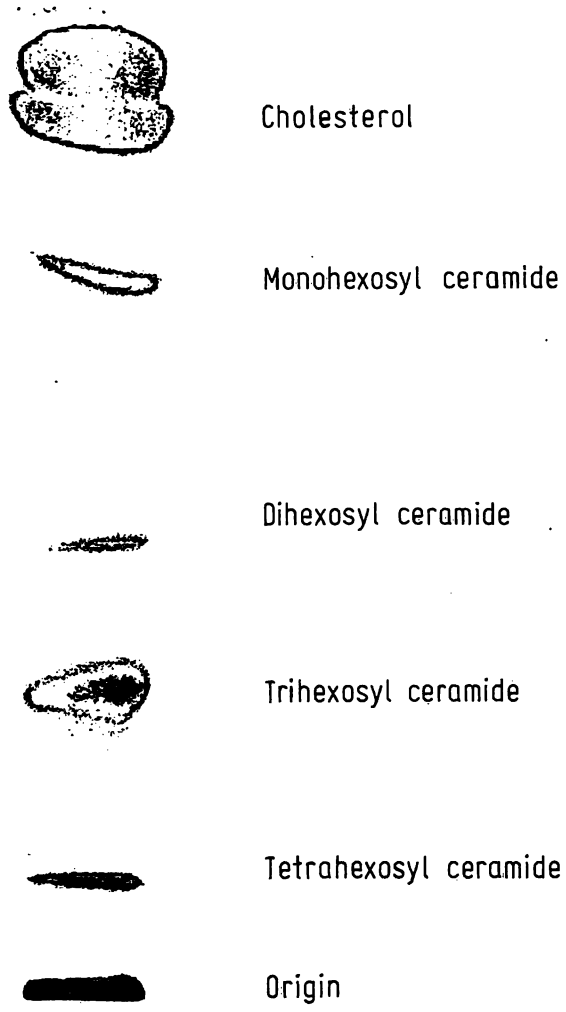



Fig. 1. Thin layer chromatogram of isolated glycosphingolipids from plasma of a patient with morbus *Fabry*. Solvent: Chloroform-methanol-water (volumes, 65 ml + 25 ml + 4 ml). Visualized with iodine vapour.

were used. Prior to use the plates were activated for 30 min at 110 °C. The samples (and reference substances) were spotted 1.5 cm from the lower edge of the plate as a 2 cm long band. The chromatogram was developed using chloroform-methanol-water (volumes, 65 ml + 25 ml + 4 ml) as the mobile phase in saturated tanks. The solvents were allowed to ascend at room temperature to a distance of 17 cm from the origin. After chromatography the plates were dried at room temperature. The lipid spots were visualized by iodine vapour (fig. 1). Rectangular fields enclosing the spots and corresponding blank zones were marked with a fine spatula. Mono-, di-, tri- and tetrahexosyl ceramides were identified by comparison with reference substances chromatographed on the same plate.

*Determination of glycosyl ceramides*

After removal of the iodine by volatilization, the marked areas of adsorbent were scraped off and transferred to glass tubes. The adsorbent pieces were minced, and 3 ml of cold orcinol reagent were added. After thoroughly mixing for 30 s with a mechanical agitator the tubes were incubated at 80 °C for 20 min. Thereafter the tubes were chilled in ice water, mixed with a mechanical agitator, and the silica gel was removed by low speed centrifugation. The absorbance was estimated at 505 nm. A sample blank was taken using 3 ml of orcinol reagent. The absorbances of the corresponding control areas were subtracted from the test samples. Aliquots of galactose (0.05, 0.1, 0.2 μmol) were treated by the same colorimetric procedure.

The hexose contents of the isolated glycosphingolipids were calculated from the standard curves, based on the relative molar absorbances of glucose and galactose (fig. 2 and 3). Galactosamine gives no reaction with orcinol (7, 8). The hexoses in the four isolated glycosphingolipids were assumed to be as follows: monohexosyl ceramide-glucose; dihexosyl ceramide – glucose-galactose; trihexosyl ceramide – glucose-galactose-galactose; tetrahexosyl ceramide – glucose-galactose-galactose-N-acetylgalactosamine (2). In control series this assumption was confirmed after trimethylsilylation of the carbohydrate moieties by gas-liquid-chromatography of the trimethylsilyl methyl glycosides (9, 10) (fig. 4). The portion of galactose in monohexosyl ceramide is about 5%.

## Results and Discussion

Table 1 represents the concentrations of glycosyl ceramides in human plasma of 23 healthy subjects. Glucosyl ceramide was found to be the predominant glycosphingolipid in normal plasma, then, in order

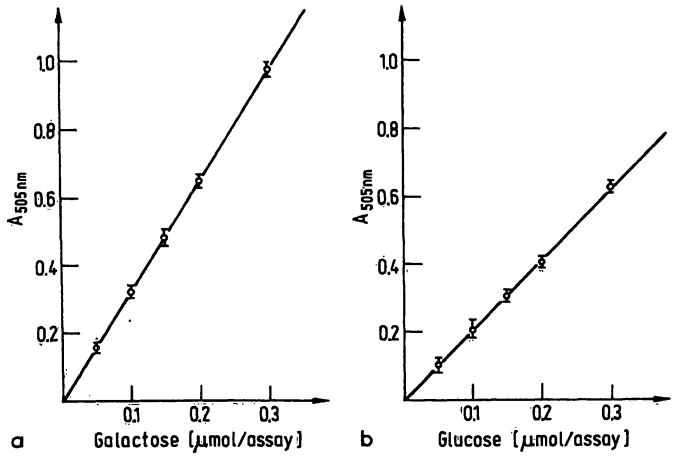



Fig. 2. Calibration curves for galactose (a) and glucose (b) determined by the orcinol reaction. The glucose curve is used for monohexosyl ceramide determination, the galactose curve for controls. Mean ± standard deviation, n = 6.

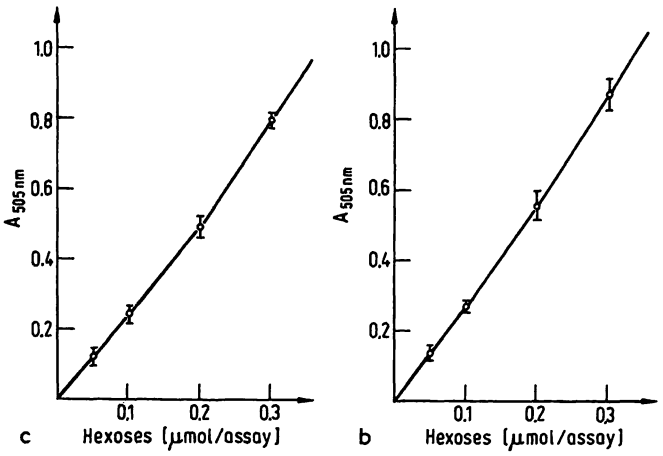



Fig. 3. Calibration curves for glucose-galactose ((a) μmol, 1 + 1) (dihexosyl ceramide) and glucose-galactose ((b) μmol, 1 + 2) (trihexosyl and tetrahexosyl ceramide), determined by the orcinol reaction. Mean ± standard deviation, n = 6.

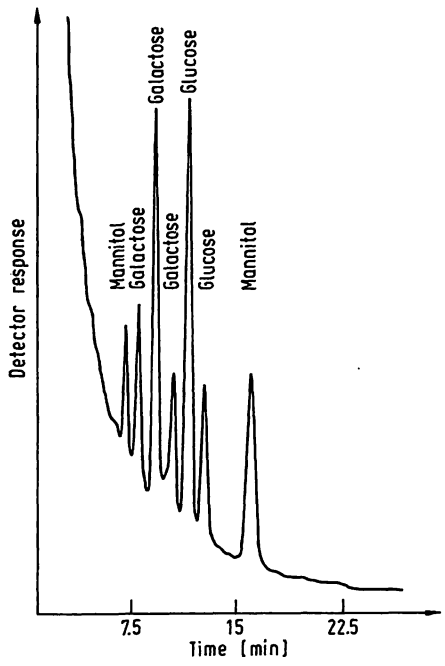



Fig. 4. Gas chromatogram of trimethylsilyl methyl glycosides of glucose and galactose from isolated plasma dihexosyl ceramide, with added mannitol (internal standard). Column of 3% SE-30 at 170 °C. Packard model 7720.

Tab. 1. Concentration of glycosyl ceramides in human plasma of healthy subjects (n = 23).
galNac = N-acetylgalactosamine, cer = ceramide.

| | $\bar{x}$ [μmol/l] | s | $s_{\bar{x}}$ |
|---|---|---|---|
| Monohexosyl ceramide (glc-cer) | 5.96 | 1.41 | 0.29 |
| Dihexosyl ceramide (gal-glc-cer) | 3.33 | 1.01 | 0.23 |
| Trihexosyl ceramide (gal-gal-glc-cer) | 1.44 | 0.45 | 0.09 |
| Tetrahexosyl ceramide (galNac-gal-gal-glc-cer) | 2.35 | 0.89 | 0.19 |

Tab. 2. Duplicate analyses of 10 ml plasma from eight individuals.
s = standard deviation.

| Donor | monohexosyl ceramide [μmol/l] | dihexosyl ceramide [μmol/l] | trihexosyl ceramide [μmol/l] | tetrahexosyl ceramide [μmol/l] |
|---|---|---|---|---|
| I | 7.6 | 2.6 | 1.3 | 1.8 |
| | 8.2 | 2.8 | 1.6 | 2.2 |
| II | 11.6 | 5.9 | 3.0 | 3.2 |
| | 11.4 | 5.3 | 2.7 | 3.5 |
| III | 5.8 | 1.5 | 1.4 | 2.0 |
| | 4.9 | 1.4 | 1.5 | 1.4 |
| IV | 3.2 | 2.4 | 1.1 | 2.0 |
| | 3.4 | 2.5 | 1.2 | 1.7 |
| V | 5.8 | 2.5 | 1.0 | 1.9 |
| | 6.2 | 2.4 | 1.2 | 2.0 |
| VI | 8.2 | 2.4 | 1.1 | 2.1 |
| | 7.0 | 2.3 | 1.1 | 2.0 |
| VII | 6.6 | 3.0 | 1.7 | 2.6 |
| | 5.8 | 2.7 | 1.5 | 1.9 |
| VIII | 5.0 | 1.8 | 0.9 | 1.3 |
| | 4.4 | 2.0 | 0.8 | 1.0 |
| s | 0.45 | 0.19 | 0.20 | 0.28 |

of decreasing concentration, dihexosyl ceramide, tetrahexosyl ceramide and trihexosyl ceramide. This is in good agreement with the results of *Vance & Sweeley* (2). Our average concentrations of plasma glycosphingolipids correspond to the values obtained by other laboratories from only few subjects and with different methods (2, 11, 12). The recoveries of added glucosyl ceramide and lactosyl ceramide, estimated according to our procedure, were 96% for glucosyl ceramide and 89% for lactosyl ceramide, respectively. The precision of the method was tested in duplicate analyses of plasma in eight individuals. The results are presented in table 2. Standard deviations were calculated by performing the analysis of variance with eight groups. They range from about 7% of the mean for mono- and dihexosyl ceramide to 13–14% for trihexosyl and tetrahexosyl ceramide.

The procedure described for the isolation and estimation of glycosphingolipids in plasma is sufficiently sensitive for routine work with 10 ml plasma aliquots. The advantage of this method in clinical investigation is the small plasma volume needed and the estimation of the carbohydrate parts without application of the irritable and time consuming procedure of gas chromatography. The method described allows the diagnostic detection of elevated sphingolipids in lipidoses like morbus *Fabry* (13) and permits further clinical investigations of glycosphingolipid metabolism (4, 5).

## Acknowledgement

The careful technical assistance of Mrs. *F. Popp* is gratefully acknowledged. We wish to thank Dipl. Math. *G. Müller* (Rechenzentrum der Universität Mainz) for performing the analysis of variance.

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